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Biodegradation of Azo Dye by *Listeria* Sp

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ABSTRACT

In this study azo dye degrading bacteria was isolated from various textile industries in and around Rajapalayam. The four bacterial strains effectively degrading the textile dyes were isolated, screened and analyzed. The toxic level of the dye and its metabolites were successfully estimated and it was found that the concentration of the protein in the culture increases with the increase in the concentration of the dye. Thus the stress is the main reason for the production of protein which in turn responsible for degradation. Phytotoxicity study was conducted in rice seed and monitoring the germination and growth rate of the seed. The results indicated that the growth of the plant is arrested when the seeds are soaked in the dye. Seed germination of the rice have a very low growth and germination rate with respect to the concentration of the dye and the degraded products showed higher in toxic level . This confirms the formation of toxic aromatic amines. Finally biochemical analysis was done for the identification of the bacterial strains responsible for the degradation of the dyes. Among the four screened isolates *Listeria* sp., was found to be more effective.

Key Words: Phytotoxicity, Seed germination, Degradation, Textile dyes and Toxin.

1. Introduction

Dyes are the coloured substances which fixes firmly with the substrate. Synthetic dyes are used extensively for textile dying, paper printing, and colour photography. Approximately 10,000 different dyes and pigments are used industrially and over 7 X 10^5 tons of these dyes are produced annually worldwide. It is estimated that 10 to 15 % of the dye is lost in the effluent during dyeing process. Dyes are broadly classified in to several types. Based on the chemical structure of chromophoric group, synthetic dyes are classified as azo dyes, anthraquinone dyes, triarylmethane dyes, etc. These dyes have an adverse effect on the environment. Disposal of Effluent in soil and river prevents the penetration of sunlight in them. These Dyes are toxic, carcinogenic and genotoxic because of their high COD values. In textile industries azo dyes play a major role in colouring process. Azo dyes are considered as electron deficient xenobiotic components because they possess azo (N=N) and sulphonic (-SO_3-)electron withdrawing groups, generating electron deficiency and making the component less susceptible to oxidative catabolism by bacteria. They are not typically degraded under aerobic conditions. Azo are mainly used in dyeing textile fibers, particularly cotton but also silk, wool, viscose and synthetic fibers. At present there are around 3,000 azo dyes in use worldwide and they account for 65% of the commercial dyes. Azo dyes are the largest group of dyes used in industry (Ramalho *et al.,* 2002) representing more than half of the annual production (Stolz, 2001). It has been estimated that about 10% of the dye stuff used during this dyeing processes does not bind to the fibers and is therefore released into the sewage

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treatment systems or environment (Zollinger, 1987). Cleavage of the azo bonds in the azo dyes gives aromatic amines which are more toxic and carcinogenic. The effect of various concentrations of dye on decolourization performance by using the isolated bacterial strains was studied (Humnabadkar *et al*., 2008, Mohan et al., 1999). Bacterial Degradation of azo dyes was carried out mostly anaerobically with only a few strains capable of degradation under aerobic conditions. In both cases colourless and possibly toxic aromatic amines are formed (Wong *et al*., 1996, Cripps *et al*., 1990).The isolation of soil bacteria which utilize these dyes as a sole source of carbon has proved difficult. Zimmerman isolated two bacterial strains capable of totally degrading the azo dyes carboxyorange I and II, but not the corresponding sulfo analogs orange I and II. In all cases, bacterial degradation is initiated by the reduction of the azo linkage to generate aromatic amines, which are degraded further (Zimmerman *et al*., 1982).The toxicity of the degraded product can be verified by using phytotoxicity study. It is used to demonstrate the toxicity of the biodegraded product for plant with respect to the Oryza sativa (ADT 43), the growth and germination rates were noted. The identification of those bacterial strains was done by using some biochemical tests. By referring the Bergey's manual (Holt *et al*., 1994) a series of biochemical tests were performed to identify the organisms.

2. Materials and Methods

2.1 Soil sample collection

The soil Samples used in the present study were collected in a polythene bag from textile effluent contaminant site of in and around Rajapalayam, Tamil Nadu. Samples were collected with scalpel after removing 20 cm of the surface layer of the soil. Collected soil samples were stored aerobically at 4ºC.

2.2 Isolation of Bacteria

2.2.1 Stock Sample Preparation

The soil and liquid samples were collected from the industrial effluent disposal area and stock solutions was prepared. Soil stock sample was prepared by taking 1 gm of soil sample in 100 ml of sterile water and Liquid stock sample was prepared by taking 1 ml of industrial outlet water with 99 ml of sterile water.

2.2.2 Serial Dilution

1 ml of culture was taken from the enriched soil mixture and mixed with 100ml of sterile distilled water. Then the samples were serially diluted from (10^{-1} to 10^{-9}) .Spread plate technique was followed, were 0.1 ml of sample was spreaded on the Nutrient agar plates using sterile glass rod and incubated at 37ºC. After overnight incubation, the isolated colonies which showed distinct morphological variation was selected, brought to pure culture and stored in slants for further use.

2.2.3 Morphological and Biochemical Characterization

Colony characteristics, colour, motility, gram staining, fermentation of sugars, biochemical test were performed for identification of potential isolates of bacteria. Cultural characteristics of the colony such as margin, size, shape, type of colony, nature of colony (Mucoid, Rough, Smooth) transparently etc., were studied.

2.2.4 Screening

The selected strains must be screened to know the property of degrading the dye, since they adapt to grow in the industrial effluent.

2.2.5 Medium inoculation

A loopful of selected bacterial strains were taken and inoculated into a separate culture tubes containing NB broth. The tubes were placed in the shaker and kept for overnight incubation at 37°C.

2.2.6 Inoculation of Dye

After overnight growth, 2ml of cultures were taken in separate test tube and 50ppm of the dye Black B was added to each tube. Similarly the same procedure was followed for the other dye like Red B5 and Black HFGR. The test tubes were kept in the shaker at 37°C. The NB broth containing the dye of same concentration was taken as the control.

2.2.7 Selection of organisms

After 24 hours the test tubes were taken and the results were compare with the control. The organisms containing test tubes showing decolourization were selected.

2.2.8 Biodegradability

The screened bacterial strains have the capability of degrading the dye. Their efficiency can be calculated by using this biodegradability assay. Here continuous monitoring of the absorbance with respect to the control was done using the UV Spectrophotometer (Hitachi) and then the percentage of degradation after 24^{th} and 48^{th} hour were observed.

2.2.9 Stock Preparation for Dye

The 20000 ppm stock solution was prepared for the dyes Black B, Black HFGR and Red B5 by taking 200 mg of dye and dissolved in 10 ml of the sterile water.

2.3 Procedure

The series of test tubes were inoculated with the screened bacterial strains and kept in the incubator for overnight. The Optical Density of the overnight culture was checked at 600nm. 500 ml of LB broth was sub cultured with this overnight culture such that its initial O.D is 0.05. For efficient degradation, it is essential that the number of viable cells should not exceed 10^8cells/ml. To monitor the growth of the culture, OD_{600} was observed for every 20-30 minutes until its OD reaches 0.6-1.0 (log phase). The Stock solution of the Dyes were taken and they were diluted to 50,100,150 and 200ppm respectively in separate conical flasks. To 2ml of the overnight culture 20 µl of dye stock was added to get 200 ppm concentration. Similarly the dyes were added in other tubes in different concentrations and kept in the shaker at 37°C. Wavelength scan was done for the dyes Black B, Black HFGR and Red B5 and their maximum absorbance was found to be 601,600 and 528 nm respectively. After 24 hours the 2ml culture was taken in the eppendorfs and centrifuged (Hitachi) at 4000 rpm for 5 minutes. The supernatant was taken in cuvettes and the absorbance at 528nm was observed for the culture with Black B by taking the NB Broth as blank and medium with the dye as control. Similarly another set of readings were taken after 48 hours.

By taking the absorbance of the control and sample .The percentage of degradation was calculated by the formula.

$$\text{Percentage of degradation} = \frac{\text{Control O.D} - \text{Sample O.D}}{\text{Control O.D}} \times 100$$

2.3.1 Total Protein Content Analysis

The degradation may happen due to several reasons but one of the main reasons is the proteins released by them. Therefore protein estimation was carried out by Lowry's Method.

2.3.2 Preparation of Reagents

Prepare the complex-forming reagent immediately before use by mixing the following stock solutions in the proportion 100:1:1 (by volume), respectively:

Solution A: 2% (w/v) Na2CO3 in distilled water.

Solution B: 1% (w/v) CuSO4·5H2O in distilled water.

Solution C: 2% (w/v) sodium potassium tartrate in distilled water.

And then Prepare 2N NaOH and 1N Folin reagent.

2.3.3 Preparation of protein standards

BSA was used as standard solution to quantify the amount of protein released .BSA stock was prepared by taking 30mg of BSA in 30ml of sterile double distilled water. A series of BSA standard was prepared from the stock solution in different concentrations like 0.1, 0.2, 0.3… 1.0 mg/ml.

2.3.4 Extraction of protein

The extraction of the protein can be done by using Sonication. The culture was taken in clean autoclaved falcon tubes and centrifuged at 4000 rpm for 5 minutes. After centrifugation add 2 ml of phosphate buffer solution to the supernatant and kept in ice. Sonicate the sample at 0.7 amp for five minutes. After sonication the samples were sealed and kept in the centrifuge (Hitachi) at 10000 rpm for 15 minutes at 4°Cto extract the protein contain in the supernatant.

2.4 Procedure

To 0.1 ml of sample or standard, add 0.1 ml of 2 *N* NaOH. Hydrolyze at 100°C for 10 min in a heating block or boiling water bath. Cool the hydrolysate to room temperature and added 1 ml of freshly mixed complex-forming reagent. Let the solution stand at room temperature for 10 min. Added 0.1 ml of Folin reagent, using a vortex mixer, and let the mixture stand at room temperature for 30–60 min (do not exceed 60 min). Read the absorbance at 660nm. Plot a standard curve of absorbance as a function of initial protein concentration and use it to determine the unknown protein concentrations.

2.5 Phytotoxicity Assay

The toxicity of the degraded product can be defined using the phytotoxicity assay. Here the tolerance level of the paddy plant ADT43 with respect to the toxicity of the dye and its degraded product was observed. The culture was inoculated to the LB Medium containing 50 ppm of Dye Black B and kept in the shaker overnight at 37°C. After degradation of the dye the culture was taken in the centrifuge tubes and centrifuged at 10000 rpm for 15 minutes. The supernatant was saved. Soak the seeds of Paddy ADT43 in the degraded product for overnight. Taking the seeds in the distilled water was taken as the control. Similarly the seeds were soaked in the test tubes containing dye at different concentration. After overnight the seeds were taken and placed on the Whatman filter paper kept over the Petri plate. The filter paper was moisture with the sterile water. Germination was continuously monitored day by day. After complete germination the mean of plumule and radical length of the germinated seeds were measured and the results are tabulated.

3. Results and discussion

Dye degrading bacteria was isolated from polluted soil in textile industries collected from in and around Rajapalayam. The subsurface soil at a depth of 20 cm was collected and stored in 4^0C for further use. The analysed soil samples were enriched and serially diluted by using serial dilution technique. The four colonies grown on higher dye concentration was selected for further studies. The isolated dye degrading bacteria was identified based on morphological and their biochemical characterization (Table 1). The degradability assay for four isolates were carried out in all three dyes(Black B, Black HFGR and red B5) (Table2,4and 6) and the percentage of degradation was noted in (Table3,5and7). The result indicated that Biomonas 4 showed higher percentage of degradation in all three dyes tested, followed by biomonas 3,2and1.Lowry's method of analysis of protein content was found by using the absorbance of the BSA Standard.

The unknown protein content can be quantified by plotting the optical density values in the BSA standard graph. The absorbance values of the BSA with respect to the concentration of the protein were measured using UV Spectrophotometer at 660 nm (Table 8).The quantification of the protein was done by plotting the optical density values in the BSA standard plot (Table 9). The tolerance level of the plant can be estimated by continuous monitoring of the germination rate and the growth rate of the paddy ADT43 seeds in the petriplates. Exactly 30 seeds are taken for germination in each petriplates and the germination of the seeds were tabulated (Table 10). The seeds soaked in distilled water show germination. The growth rate of the seeds soaked in the dye at different concentration is very low when compared with the control. There is no germination in the seeds soaked in the degraded products of the dye.

The bacterial strains effective in degrading the textile Dyes Black B, Black HFGR and Red B5 were isolated from the industrial effluent samples. It has been isolated, since it has adaptability towards the dye because it exists in the stress. Twelve morphologically different colonies from the effluent sample were isolated. The isolated bacterial strains were then screened by growing then in medium containing dye. The degradation of the dye was monitored and the organisms which effectively degrading the dye was selected and sub cultured in slants for further use. Among the twelve isolated strains four of them are effective in degrading the dyes. The screened organisms were then analyzed using Biodegradability test to find out its efficiency in degrading the dyes Black B, Black HFGR and Red B5.the absorbance values were measured using UV Spectrophotometer and the percentage of degradation for was calculated for each dye by four different screened organisms. By analyzing the results it is clearly seen that the organism 2 is the most effective strain. Then the protein content of the culture was analyzed by Lowry's method with Bovine Serum Albumin fraction as the standard. By plotting with standards the concentration of the protein in the culture is estimated. The

results showed that the concentration of the protein increases with the increase in concentration of the dye. Thus it clearly indicated, that the stress (Dye) shows the influence of the protein content in the culture. The degradation of the azo dyes results in formation of the aromatic amines. These aromatic amines are highly toxic and carcinogenic. The toxicity level can be estimated by monitoring the germination and growth rate. From the results, growth of the plant is arrested when the seeds are soaked in the dye. It has a very low growth and germination rate with respect to the concentration of the dye and the degraded products show a higher toxic level so that no germination was seen on those seeds soaked in the degraded products of the dye. This confirms the formation of toxic aromatic amines.

Table 1: Summary of Biochemical Tests Based on the reference with the Bergey's Manual the Organism 4 was found to be *Listeria* sp.

Test	**Biomonas 1**	**Biomonas 2**	**Biomonas 3**	**Biomonas 4**
Gram Staining	+	-	+	+
Morphology	Irregular rods, single often double.	Cocci in short chains, Mostly diplococci	Irregular rods, single, short rods	Regular rods, short chains and filaments
Endospore Formation	-	-	-	-
Motility	Non motile	Motile	Motile	Motile
Catalase Test	-	+	+	+
Indole Test	-	-	-	-
Simmons Citrate Test	-	-	-	-
Acid From Carbohydrate	+ (oxidative)	+ (fermentative)	+ (fermentative)	+ (fermentative)

Table 2: The degradability assay for the dye Black B

Dye conc. ppm	**Control**	**Degradability assay for Black B**							
		Biomonas 1		**Biomonas 2**		**Biomonas 3**		**Biomonas 4**	
		24h	48h	24h	48h	24h	48h	24h	48h
50	0.877	0.46	0.36	0.353	0.213	0.46	0.326	0.333	0.265
100	1.207	0.864	0.526	0.905	0.623	0.921	0.575	0.778	0.517
150	1.805	1.273	0.835	0.961	0.718	1.515	0.968	1.392	0.923
200	2.203	2.145	1.825	1.235	0.964	1.986	1.125	1.749	1.513

Table 3: Percentage of Degradation of Black B by screened organisms

Dye Conc. ppm	Percent degradation of Black B over control							
	Biomonas 1		Biomonas 2		Biomonas 3		Biomonas 4	
	24h	48h	24h	48h	24h	48h	24h	48h
50	47.548	58.951	59.749	75.713	47.548	62.828	62.030	69.783
100	28.418	56.421	25.021	48.384	23.695	52.361	35.543	57.167
150	29.474	53.740	46.759	60.222	16.066	46.371	22.881	48.864
200	2.633	17.158	43.940	56.241	9.850	48.933	20.608	31.321

Table 4: The degradability assay for the Dye Black HFGR

Dye Conc. ppm	Control	Degradability assay for Black HFGR							
		Biomonas 1		Biomonas 2		Biomonas 3		Biomonas 4	
		24h	48h	24h	48h	24h	48h	24h	48h
50	0.867	0.394	0.271	0.398	0.271	0.338	0.168	0.421	0.221
100	1.39	0.811	0.65	0.803	0.36	0.981	0.583	1.021	0.56
150	1.959	1.543	1.088	1.45	1.023	1.654	1.101	1.451	0.82
200	2.25	2.076	1.964	1.979	1.139	1.845	1.683	1.65	1.021

Table 5: Percentage of Degradation of Black HFGR by screened organisms

Dye conc. ppm	Percent degradation of Black HFGR over control							
	Biomonas 1		Biomonas 2		Biomonas 3		Biomonas 4	
	24h	48h	24h	48h	24h	48h	24h	48h
50	54.556	68.743	54.095	68.743	61.015	80.623	51.442	74.510
100	41.655	53.237	42.230	74.101	29.424	58.058	26.547	59.712
150	21.235	44.461	25.983	47.779	15.569	43.798	25.932	58.142
200	7.733	12.711	12.044	49.378	18.000	25.200	26.667	54.622

Table 6: The degradability assay for the Dye Red B5

Dye Conc. ppm	Control	Degradability assay for Red B5							
		Biomonas 1		Biomonas 2		Biomonas 3		Biomonas 4	
		24h	48h	24h	48h	24h	48h	24h	48h
50	0.608	0.369	0.112	0.339	0.424	0.554	0.491	0.196	0.18
100	1.289	1.142	0.416	1.162	0.684	1.006	0.797	0.925	0.439
150	1.79	1.641	1.073	1.379	0.797	1.654	1.478	1.215	1.25
200	2.204	1.68	1.51	1.673	1.336	1.807	1.72	1.715	1.318

Table 7: Percentage of Degradation of Red B5 by screened organisms

Dye Conc. ppm	Percent degradation of Red B5 over control							
	Biomonas 1		Biomonas 2		Biomonas 3		Biomonas 4	
	24h	48h	24h	48h	24h	48h	24h	48h
50	39.309	81.579	44.243	30.263	8.882	19.243	67.763	70.395
100	11.404	67.727	9.853	46.936	21.955	38.169	28.239	65.943
150	8.324	40.056	22.961	55.475	7.598	17.430	32.123	30.168
200	23.775	31.488	24.093	39.383	18.013	21.960	22.187	40.200

Table 8: Standard BSA plot values

Concentration of BSA (mg/ml)	Optical density at 660nm
0.1	0.158
0.2	0.264
0.3	0.369
0.4	0.491
0.5	0.621
0.6	0.671
0.7	0.77
0.8	0.894
0.9	0.954
1	0.988

Table 9: Total protein content in culture containing Black B after 24 hrs of Degradation

Organism	Concentration of Dye (ppm)	Optical Density at 660nm	Conc. of protein (mg/ml)
	without dye	0.346	0.352
	50	0.416	0.421
	100	0.572	0.595
Biomonas 1	150	0.642	0.680
	without dye	0.387	0.394
	50	0.452	0.498
	100	0.766	0.790
Biomonas 2	150	0.792	0.821
	without dye	0.382	0.411
	50	0.423	0.452
	100	0.660	0.695
Biomonas 3	150	0.694	0.726
	without dye	0.442	0.465
	50	0.703	0.743
	100	0.79	0.820
Biomonas 4	150	0.83	0.863

Table 10: Phytotoxicity study of Black B and its metabolites produced after decolourization

Sample	No of Germinated seeds	Germination Rate (%)	Mean plumule length (cm)	Mean radical length (cm)
Distilled water	29	100	6.8	4.2
Dye (50 ppm)	19	65.5	4.9	3.5
Dye (100 ppm)	13	44.8	4.1	3.1
Degraded product of Org1	0	0	0	0
Degraded product of Org2	0	0	0	0
Degraded product of Org3	0	0	0	0
Degraded product of Org4	0	0	0	0

The biochemical tests were done to identify the bacteria responsible for degradation of dye. First step in the biochemical analysis is the gram staining. Using this we can differentiate wide species of bacteria in two groups i.e., gram positive and gram negative. Then the morphological characters of the strains were observed under the microscope. The motility is also a major factor in

differentiating the bacterial strains. By doing the several other biochemical tests genus of bacteria can be identified. Among the four bacterial strains biomonas 4 was identified as *Listeria* sp., which showed higher degradation of the dye.

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